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(54) Method for the preparation of a protein hydrolyzate from whey protein.

(57) Disclosed is a process for the preparation of a protein hydrolyzate suitable for use in an enteric diet. The method involves the enzymatic hydrolysis of whey protein (particularly lactalbumin) using foodgrade fungal protease produced by an organism of the species *Aspergillus oryzae*.

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1 METHOD FOR THE PREPARATION OF A
 PROTEIN HYDROLYZATE FROM WHEY PROTEIN

BACKGROUND OF THE INVENTION

 Enteric diets, i. e., those diets which comprise
5 nutrients which are designed to pass through the stomach un-
 altered and be absorbed by the intestines, are necessitated by
 various disorders. A suitable enteric material must, of
 course, contain amino acids to provide complete nutrition.

 It does not seem necessary that an elemental enteric
10 diet contain only amino acids. Rather, recent evidence sug-
 gests that peptides 2-3 units long are absorbed even more
 readily than the individual amino acids in some cases. The
 original theory of protein absorption was that small peptides
 liberated by the pancreatic proteases were hydrolyzed to their
15 constituent amino acids by brush border peptidases. These
 amino acids are then transported across the cell membrane
 by an active transport system coupled to the sodium pump.
 It now appears that in addition to this there is a mechanism
 specific for the uptake of small peptides. Di- and tri-
20 peptides are actively transported against concentration
 gradients by a common mechanism and are later hydrolyzed by

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1 cytoplasmic peptidases. Longer peptides are hydrolyzed at
the brush border membrane with absorption of the resultant
amino acids or di- to tri- peptides. The rates of absorption
of peptides are frequently faster than those of the free amino
5 acids, and the peptides are absorbed well in the proximal and
distal small intestine, while free amino acids are absorbed
most readily in the proximal region.

There is also evidence that peptide diets may be useful
in the treatment of amino acid absorption diseases. In
10 Hartnupp's disease, transport of neutral amino acids is di-
minished but the transport of peptides is unaffected. In
cystinuria, small intestinal absorption of cysteine, ornithine,
arginine and lysine are diminished but again the absorption of
amino acids is normal if they are administered as di- and tri-
15 peptides. In Lowe's syndrome, there is a decrease in all
amino acid transport although peptide transport is not affected.
In fact, there is no evidence of a primary peptidase deficiency
or a peptide transport deficiency analogous to that of amino
acids.

20 Malabsorption of amino acids is associated with many
small intestinal disorders whereas dipeptide absorption is less
severely affected. In tropical and celiac sprue, absorption
of free amino acids is reduced while dipeptide absorption is
fairly normal. In jejunioleostomy for obesity, a reduction of
25 the absorption of free amino acid leucine occurs without any
reduction in the absorption of the dipeptide glycyl-leucine.

Diets of free amino acids are hyperosmotic as compared
to peptide diets and it has been shown that a peptide diet causes
less fluid secretion into the small intestine than an equal
30 nitrogen content amino acid diet.

1 In view of the above discussion, it is apparent that an
elemental enteric diet containing amino acids and peptides
would be preferable to one containing only amino acids. Op-
timally the peptides should contain from 2 to 3 amino acid
5 residue groups. While the optimal 2 to 3 group peptide may
not be achievable on a commercial scale at a reasonable cost,
a material containing a substantial amount of di- and tri-
peptides along with some amino acids and higher molecular
weight polypeptides is suitable for use in an enteric diet. The
10 di- and tri- peptides would be transported intact, whereas, the
tetra-, penta and hexa- peptides would be hydrolyzed by pri-
mary brush border peptidases and the resulting di- and tri-
peptides transported across the cell membrane. Amino acids
would be absorbed in some cases and excreted in others, de-
15 pending on the malady involved. Higher molecular weight
polypeptides would be excreted. Thus, a protein hydrolyzate
suitable for use in an enteric diet will desirably contain at
least 50 weight percent of a combination of amino acids, di-
peptides and tri- peptides and not more than 25 weight percent
20 of polypeptides containing 10 or more amino acids:

Amino acids and peptides used in elemental enteric
diets may be prepared by the enzymatic hydrolysis of a pro-
tein source material. In view of the above discussion, it can
be seen that control of the molecular weight distribution of a
25 protein hydrolyzate for use in such a diet is essential.

The flavor of its protein hydrolyzate is also a major
factor in the success of an elemental enteric diet. Thus, it
is desirable not only to produce a protein hydrolyzate with the
proper molecular weight distribution but also one with a bland
30 flavor. The protein source can have a major effect on the

1 flavor of a hydrolyzate produced from it. Legumes such as
soybeans are notorious for their bitter, grassy, burnt, catty
and fusel notes. Many of the compounds responsible for these
flavors (long chain alcohols, ketones and aldehydes) are com-
pounds of the raw bean and decrease on heating, but new ones
5 develop. Further processing is necessary to remove these
materials.

Fish protein concentrates also present problems.
Fish protein is usually contaminated with 1°, 2° and 3° amines
10 which contribute significantly to its characteristic flavor. In
addition, fish muscle contains from 1-16% fat which is poly-
unsaturated. These lipids are difficult to extract and are
readily oxidized by air or lip^{f} oxygenases and esterases (present
in the fish flesh) to objectionably flavored compounds. The
15 protein hydrolyzate itself presents a flavor problem since
many amino acids, especially the more hydrophobic ones, are
themselves bitter. Although the particular enzyme applied in
the hydrolysis exerts some effect on the level of bitterness,
the protein itself is also a factor.

20 It is also required that the protein hydrolyzate have a
protein efficiency ratio (PER) at least equal to that of whole
egg, i. e., at least 2.5. For this reason, and because of its
bland flavor, we prefer to use whey protein (PER of 3.0) as
the starting material for our hydrolyzate. In the production
25 of cheese, milk solid, i. e., casein, is precipitated from milk
either by acid precipitation or enzymatic coagulation leaving
a liquid phase containing whey proteins. The solid whey pro-
tein can be recovered by various techniques such as heat pre-
cipitation, reverse osmosis, gel filtration and electrodialysis.
30 We prefer to use a whey protein obtained by heat precipitation
(lactalbumin).

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1 Lactose, which is a contaminant of most whey proteins is not digested by a large proportion of the population and is a common cause of gastrointestinal complaints. Therefore, it must be present only in very low levels, if at all, in
5 the hydrolyzate. The amount of lactose that produces symptoms has been investigated, and based on the investigations it can be concluded that whey protein used as the starting material for a protein hydrolyzate should contain a level of lactose such that the hydrolyzate produced from it will contain no
10 greater than 1.0 weight percent of the sugar.

SUMMARY OF THE INVENTION

The present invention is a method for the preparation of a protein hydrolyzate suitable, both in terms of molecular weight profile and flavor, for use in products to be consumed
15 by individuals on an enteric diet. The method comprises the steps of:

- a) providing whey protein having a lactose level sufficiently low to provide a protein hydrolyzate containing no more than 1.0 weight percent of this sugar;
- 20 b) forming an aqueous slurry of the whey protein;
- c) adding foodgrade neutral fungal protease from Aspergillus oryzae to the slurry in an amount of from about 18.9 to 189 spectrophotometric hemoglobin units per gram of whey protein;
- 25 d) maintaining the pH and temperature of the slurry containing the protease at a level of from about pH 3.0 to about 10.0 and from about 40° C. to about 70° C. for a time sufficient to hydrolyze the whey protein into a hydrolyzate containing at least 50 weight percent of a combination of amino

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- 1 acids, di-peptides and tri-peptides and not more than 25
weight percent of polypeptides containing 10 or more amino
acids;
- e) heating the slurry to a temperature and for a time
5 sufficient to inactivate the enzyme;
- f) removing remaining solid material from the slurry
to provide an aqueous solution containing the desired protein
hydrolyzate; and
- g) recovering the protein hydrolyzate from the
10 solution.

DETAILED DESCRIPTION

The first step of this method involves the procurement of low lactose whey protein. Since we prefer to use lactalbumin as the protein source, the following discussion
15 will be directed to the use of this particular whey protein. The lactose level of lactalbumin can be minimized by washing the sugar from the heat precipitated curd either before or after it is dried. When lactalbumin prepared in this manner is unavailable, lactose can be removed by hydrolysis with lactase.

20 Experiments involving the formation of hydrolyzates from enzymatically hydrolyzed lactalbumin were carried out using the following enzymes:

1. Fungal protease; obtained by controlled fermentation of Aspergillus oryzae var. The enzyme preparation contains a mixture of acid neutral and alkaline proteases exhibiting
25 activity from pH 3.0 to 10.0 but having a maximum at pH 9.0. The preparation exhibits proteolytic activity to 70° C., but has a maximum at 55° C. In the examples, we used an enzyme preparation -- Takamine Brand Fungal Protease -- from Miles

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- 1 Laboratories, Inc. which had an activity of 3,780 Spectro-
photometric Hemoglobin Units (SHU) per gram. One SHU is
that activity which will liberate one micromole of tyrosine per
minute under the conditions of the assay according to the ap-
5 proved methods of the American Association of Cereal
Chemists, 1969; Proteolytic Activity - Spectrophotometric
Method (AACC method 22-63); American Association of Cereal
Chemists, St. Paul, Minnesota.

2. Bacterial protease; obtained by controlled fermenta-
10 tion of Bacillus licheniformis var. The enzyme preparation
contains primarily endopeptidases and exhibits proteolytic
activity from pH 3.0 to 9.0 with a maximum from 5.0 to 5.5.
The enzyme preparation is active up to 70° C., with a max-
imum at 55° C. We used an enzyme preparation -- Alcalase
15 0.6L -- from Novo Industri A/S, Bagsvaerd, Denmark, having
an activity of 628 SHU/gm.

3. HT Proteolytic concentrate, Papain 3,000 and Pan-
creatin 4NE, all produced by Miles Laboratories, Inc., were
also tested under their optimal conditions.

- 20 All of the resulting protein hydrolyzates were evaluated
for flavor, molecular weight distribution and amino acid pro-
file. The fungal protease concentrate was judged as the pre-
ferred material for the production of lactalbumin hydrolyzate
to be used in enteric diets. The other enzyme preparations
25 failed primarily due to unpleasant product flavor and less de-
sirable peptide size of the protein hydrolyzate.

1 Although the fungal protease concentrate was found to
 be the best protease preparation for lactalbumin hydrolysis
 in respect to flavor and peptide size, one drawback in using
 this enzyme was found to be a low hydrolyzate yield of about
 5 40%. To increase the accessibility of substrate to enzyme,
 the lactalbumin slurry was subjected to acid (30 minutes
 boiling in 2% H_2SO_4 solution) or alkali (10 minutes boiling at
 pH 8.0) prior to enzymatic hydrolysis. The results of this
 experiment are set out in Table II.

10

TABLE IIEffect of Heat Treatment on Hydrolysis Yield

	Lactalbumin Treatment	Enzyme Level (% w/w)	Hydrolysis Conditions	Yield (%)	ApL [*]
15	Control	1.0	pH 7.0, 50° C, 7 hr.	39.6	2.3
	Acid-Heating	1.0	pH 7.0, 50° C, 7 hr.	28.8	3.2
20	Alkali-Heating	1.0	pH 7.0, 50° C, 7 hr.	58.2	2.4

* Average peptide length

The average peptide length is the ratio of amino nitrogen
 to total nitrogen. Amino nitrogen was determined by trinitro-
 25 benzene sulfonic acid (TNBS) according to the description of
 Adler Nissen (J. Ag. Food Chem., 27:1256, 1979). Total nitro-
 gen was determined by the Kjeldahl method:

1 From Table II it can be determined that the alkali-heat
treatment of lactalbumin rendered the protein more accessible
to protease attack, thereby increasing the yield, without sig-
nificantly affecting the average peptide length of the hydroly-
5 zate.

Lactalbumin was screened into different particle sizes
(50, 100 and 200 mesh) after being treated with alkali and
hydrolyzed with 1% fungal protease concentrate for 7 hours
at pH 7.0 and 50° C. The results are set out in Table III.

10

TABLE III
Effect of Particle Size on Hydrolysis Yield

	Particle Size (Mesh)	Yield (%)
15	50	57.3
	100	58.7
	200	55.8

From Table III it can be determined that particle size of
lactalbumin does not significantly affect the degree of
20 hydrolysis.

Sensory evaluation of lactalbumin hydrolyzates prepared
with various enzymes showed that 1% fungal protease or 1%
fungal proetase combined with 1% Alcalase yielded the product
having the least flavor. Hydrolyzates prepared with 10%
25 Alcalase were extremely bitter, especially when digested under
alkali conditions. Two percent (2%) Pancreatin 4NE also pro-
duced bitter hydrolyzates, intermediate between fungal

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1 protease and Alcalase. Ten percent (10%) Alcalase or a
combination of 1% fungal protease with 10% Alcalase was ex-
tremely bitter. Miles Laboratories, Inc. HT Proteolytic
concentrate and Papain 3,000 also produced a bitter hydroly-
5 zate. Depending on the various reaction conditions such as
temperature, pH and protease concentration, the hydrolysis
is typically carried out for a period of 2 to 50 hours.

Using 1% fungal protease, hydrolyzates were prepared
with different antimicrobial agents (200 ppm sulfite or 1%
10 toluene). Neither of these samples were significantly dif-
ferent in flavor from the control. During the alkaline heating
step and the hydrolysis reaction, a base is added for pH con-
trol. We prefer to use a base other than NaOH in order to
keep the sodium content low. $\text{Ca}(\text{OH})_2$ is the preferred base
15 and no difference in flavor was noted when either NaOH or
 $\text{Ca}(\text{OH})_2$ was used for pH control.

The method of practicing the method is further illus-
trated by the following example:

EXAMPLE I

20 Lactalbumin (35 Kg obtained from New Zealand Dairy)
was combined with 180 gallons of deionized water in a 200
gallon kettle equipped with a steam jacket to form a 5% (w/v)
aqueous slurry. Approximately 18 liters of a 4% NaOH solution
was added to raise the pH to 7.0 whereupon the temperature
25 was increased to 60° C. and the slurry stirred for 15 minutes.
The resultant was centrifuged using a Westfalia Separator (bowl
speed 6500 RPM, Model 9AMRCO36) to provide 60 gallons of
sludge and 130 gallons of supernatant containing about 0.5%
solids which was discarded. An additional 130 gallons of

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- 1 deionized water was added to the sludge to form a 5% (w/v)
aqueous slurry which was adjusted to pH 7.0, stirred for 15
minutes at 60° C., centrifuged and separated as before to
provide 60 gallons of lactalbumin sludge having a lactose
5 level below 1:0 (w/w) based on the dry lactalbumin. An ad-
ditional 30 gallons of deionized water was added to provide
slurry containing about 10% (w/v) solids.

- The lactalbumin slurry was adjusted to pH 8.0 by the
addition of 4.6 liters of a 4% NaOH solution whereupon the
10 slurry was heated to 90-95° C. for 10 minutes with stirring
and then cooled to 50° C. With the pH of the slurry controlled
at 7.0 by the addition of approximately 200 grams of lime in a
10% slurry, 300 grams (3780 SHU/gm.) of Miles fungal pro-
tease dissolved in 10 liters of water was added to the slurry.
15 The slurry was maintained at 50° C. for 24 hours whereupon
it was heated to 90° C. for 5 minutes, then cooled to 50° C.
The cooled slurry may be clarified by either centrifugation or
filtration with a commercially available filter aid. The clari-
fied hydrolyzate may be mixed with the remaining ingredients
20 of the enteric diet (i. e., carbohydrates, fat, vitamins and
minerals) and then dried or dried first and then blended with
the remaining ingredients. A hydrolyzate prepared according
to the instructions of this example should have an average
peptide length of 2.3. Lactose content of the lactalbumin hy-
25 drolyzate was measured using a Lactose Assay Kit from
Boehringer Mannheim (Indianapolis, Indiana). In it, lactose
is hydrolyzed to glucose and β -galactose in the presence of
 β -galactosidase and water. β -galactose is oxidized by
nicotinamide adenine dinucleotide (NAD) to galactonic acid in
30 the presence of the enzyme β -galactose dehydrogenase and

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- 1 NAD is converted to NADH. The amount of NADH formed is stoichiometric with the amount of lactose. The increase in NADH is measured by means of its absorption at 334, 340 or 365 nm. The lactose level of the hydrolyzate prepared in this example was less than 0.5% (w/w) which means that the formulated diet will contain less than about 0.05% lactose.

EXAMPLE II

- The procedure of Example I was repeated except that after 7 hours of digestion with 1% fungal protease an additional 1% of enzyme was added and the digestion continued for an additional 7 hours to provide a protein hydrolyzate having an average peptide length of 2.3.

EXAMPLE III

- The procedure of Example II was repeated except that 1% Alcalase was added after 7 hours to provide a protein hydrolyzate having an average peptide length of 2.3.

EXAMPLE IV

- The molecular weight profiles of the hydrolyzates prepared in Examples I and II were determined by exclusion chromatography according to the method of Carnegie, Nature, 206:1128, 1965. The gel selected for this was Sephadex G-25, particle size 20-80 μ . Column dimensions were 90 x 1.5 cm. and the solvent was phenol:acetic acid:water, 1:1:1, wt.:wt.:vol. A calibration curve was determined and elution volumes were calculated for amino acids to peptides of three units long, peptides from 4 to 9 amino acids long and peptides of 10 or

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1 more amino acids. Samples of hydrolyzate from Examples
I and II were evaluated for molecular weight profile by de-
termining the percent nitrogen present in those three fractions
of the column eluent. This method, which has a margin of
5 error of $\pm 3\%$ indicated that the molecular weight profile of
the samples analyzed was 65% amino acids and di- and tri-
peptides; 20% polypeptides of 4 to 9 amino acids and 16% of
polypeptides containing 10 or more amino acids.

WHAT IS CLAIMED IS:

- 1 1. A process for preparing a protein hydrolyzate suitable for use in products to be consumed by individuals on an enteric diet, which process comprises the steps of:
- 5 a) providing whey protein having a lactose level sufficiently low to provide a protein hydrolyzate containing no more than 1.0 weight percent of this sugar;
- b) forming an aqueous slurry of the whey protein;
- c) adding foodgrade neutral fungal protease from Aspergillus oryzae to the slurry in an amount of from about
- 10 18.9 to 189 Spectrophotometric Hemoglobin Units per gram of whey protein;
- d) maintaining the pH and temperature of the slurry containing the protease at a level of from about pH 3.0 to about 10.0 and from about 40° C. to about 70° C. for a time sufficient
- 15 to convert the whey protein into a hydrolyzate containing at least 50 weight percent of a combination of amino acids, di-peptides and tri-peptides and not more than 25 weight percent of polypeptides containing 10 or more amino acids;
- e) heating the slurry to a temperature and for a time
- 20 sufficient to inactivate the enzyme;
- f) removing remaining solid material from the slurry to provide an aqueous solution containing the desired protein hydrolyzate; and
- g) recovering the protein hydrolyzate from the solution.
- 25 2. The process of Claim 1 wherein the whey protein is lactalbumin.

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- 1 3. The process of Claim 1 wherein the pH is about 9.0.
4. The process of Claim 1 wherein the temperature is
about 55° C.
- 5 5. The process of Claim 1 wherein in addition to the
fungal protease there is added to the slurry a bacterial pro-
tease obtained by the controlled fermentation of Bacillus
licheniformis and the pH is maintained at a level of from 3.0
to 9.0.
- 10 6. The process of Claim 2 wherein the lactalbumin is
heated in an alkaline solution prior to the enzymatic hydrolysis.
7. The process of Claim 1 wherein the enzymatic
hydrolysis is carried out for a period of from 2 to 50 hours.
8. The process of Claim 1 wherein the pH of the slurry
is controlled by the use of $\text{Ca}(\text{OH})_2$.



European Patent
Office

EUROPEAN SEARCH REPORT

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Application number

EP 82 10 3656

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
X	LU-A- 40 694 (HOWARD LLOYD) * Claims 1,2,5; example 1 *	1,2,4,7	A 23 J 3/00
A	US-A-3 761 353 (F.F. NOE, W.T. FAITH) * Claims 1-3 * -----	1,5	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 7)
			A 23 J
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 31-08-1982	Examiner PEETERS J.C.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, r after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

NT-3 gene was cloned from mouse, rat, and human (see U.S. patent application Ser. No. **07/490,004**, filed Mar. 7, 1990, incorporated by reference in its entirety herein)